Progress Report

of Work Done under Navy Contract N6-ONR-26007

from

January 1, 1953

to

June 30, 1953

Respectfully submitted by

Principal Investigator

Assistant Dean, University
of Kansas Graduate School

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Outline for
OFFICE OF NAVAL RESEARCH
Microbiology Branch

SEMI-ANNUAL PROGRESS REPORT

Report prepared by: Dr. Cora M. Downs

Date: July 8, 1953
For period: January 1, 1953, to June 30, 1953

NR:
Contract: N6-ONR-26007
Annual Rate: $ 20,928.30
Contractor: University of Kansas
Principal Investigator: Cora M. Downs, Ph.D.
Assistants: See under Personnel
Title of Project: Studies on the Pathogenesis and Immunity of Tularemia
Objectives: The study of the mechanism of immunity in tularemia by means of an attempt to isolate an immunogenic antigen and to understand the role of circulating antibodies and tissue cells in the immune animal.

Abstract (or Summary of Results)

a. Since start of project: (see following pages)

b. During current report period

Plans for Future:

Immediate: Continue a study of tissue and phagocytic reactions in immune mice as compared to normal mice, and continued attempts to isolate a protective antigen.

Long range: See next page.

Reports and Publications:

1. Studies on the pathogenesis and immunity of tularemia. I. The demonstration of a protective antibody in mouse serum. The Journal of Infectious Diseases, Vol. 92, pp. 195-204, 1953.
2. Three papers to be published from the thesis submitted by Max Moody.
3. One paper to be published on the clearance tests in mice and rats. Abstract published Proceedings of the Society, May, 1952, pp. 79-80.

4. One paper to be published on the relation of circulating antibodies to immunity.

LONG-RANGE OBJECTIVES

The problems of immunity in tularemia pose many intriguing questions. There is a great difference in host response as indicated by the solid immunity produced in white rats by killed vaccines and the almost complete lack of immunity which may be induced by these same vaccines in white mice, rabbits, guinea pigs, cotton rats, and other animals. The latter animals becomes immune to varying degrees when injected with living organisms of lowered virulence. This may indicate that the multiplying organisms in the infected animal produce an immunogenic antigen or that the immunogenic antigen for the white mouse and other animals is highly labile, and that rats and more resistant animals respond to other antigens available in killed cultures. The elucidation of this question of immunogenic antigen is one of the long-range objectives of this report.

It has been shown in this laboratory that immune animals dispose of large numbers of virulent organisms in a few days after challenge. The summary of work reported in the past reports gives evidence that the amount of mouse protection shown by antibodies from recovered and vaccinated humans, rabbits, rats, and mice is minimal and that the amount of protection shown cannot account for the immunity to challenge exhibited by humans, rats, and mice.

Further studies on blood clearance have confirmed earlier work and have indicated the importance of tissue phagocytes in immunity. This work is being extended to include a study of phagocytic mechanisms.

Studies in this laboratory and at Camp Detrick have shown that dissociation occurs in cultures of Bacterium tularense and the studies on dissociation performed in this laboratory are embodied in a thesis presented by Max Moody in partial fulfillment of the degree of Doctor of Philosophy. These studies suggest the presence of an antigen having immunogenic properties which is present in some smooth, partially virulent or virulent strains, and which is lacking in non-smooth or in some smooth avirulent strains; smooth strains of 38 are an example of the latter. Studies should be carried on and extended to elucidate the nature of this smooth antigen associated with virulence.

STUDIES ON THE PATHOGENESIS AND IMMUNITY OF TULAREMIA

SUMMARY
July 1, 1951, through December 31, 1951

Contract N6-ONR-26007
University of Kansas, Lawrence

The experiments which are summarized fall into 3 general categories: I. Antigen Studies, II. Immunity Studies, and III. Variation Studies.

I. Antigen Studies

1. Progress has been made in growing large quantities of Bacterium tularense by using concentrated media in a cellophane sack suspended in distilled water. The cells have been used for the extraction of polysaccharide and are to be used for other types of extraction.

2. Sterile tissue extracts from infected animals have been used in preliminary experiments and have indicated the presence of an immunizing antigen.

II. Immunity Studies

3. A protection test in mice has been developed which appears to be reliable.

4. The results of protection tests appeared to show that the serum from recovered animals was more protective than from vaccinated animals.

5. There was no correlation between protective, agglutinative, or hemagglutinative antibodies.

6. A satisfactory bactericidal test has been worked out.

7. This test indicates that whole blood from rabbits vaccinated with killed cultures is more bactericidal than blood from normal rabbits.

8. Blood from recovered or recently vaccinated persons is more bactericidal than blood from normal persons or those vaccinated a number of months

before the test. Bacteriostatic effect might be a better term than bactericidal, since the blood is rarely sterilized and only small numbers of bacteria are disposed of.

III. Variation Studies

9. Variants of the partially virulent Jap strain are shown to vary in toxicity for white mice when injected in large numbers in the living state.

10. Variants of the Jap strain have been shown to vary in antigenicity as tested by the agglutination test after injection into rabbits. The smooth strain showed a greater degree of antigenicity than the non-smooth strain. This was in agreement with the findings reported earlier in white mice.

SUMMARY

Period of report, January 1, 1952, to June 30, 1952

Antigenic Studies:

1. Polysaccharides from a virulent and partially virulent strain have been prepared and partially characterized.

2. These polysaccharides are not immunogenic for white mice.

3. Antigens isolated from cold macerated tissues from infected mice prolong life when injected into normal mice, but do not enable them to resist challenge with virulent organisms.

Immunity Studies:

1. A protective test has been worked out and evaluated.

2. The protective power of rabbit serum for mice has been shown to be greater from recovered rabbits than from vaccinated rabbits.

3. Bactericidal tests on human and rabbit whole blood have shown that blood from recovered humans and rabbits is more bacteriostatic than from vaccinated humans or rabbits.

4. A series of tests on Ascoli antigens are reported. These tests have shown that the antigen appears in mice as soon as abundant proliferation of the organism occurs and is generally lacking in challenged immune animals.

5. The Ascoli antigen apparently is not immunogenic for white mice.

6. Clearance tests on white mice show that the actively or passively immune mouse removes living cells from the blood stream with great rapidity and concentrates them in the liver. The implications of this mechanism are discussed.

Variation Studies:

1. A summary of the work on variants shows that the smooth and non-smooth strains can be divided into two distinct groups on the basis of their respective properties.

2. Experiments are cited which show that non-smooth strains do not produce as high a titer of agglutinins in rabbits as the smooth strains and neither is immunogenic.

3. The non-smooth strains produce a lower titer of agglutinins in rats than the smooth strains but are immunogenic.

4. No *in vivo* dissociation of smooth strains has been observed in normal or immune rats or mice.

Abstract of Report

July 1, 1952, to December 31, 1952

1. Mouse protection and mouse neutralization tests have been used to assay antibodies in mouse, rat, human, and rabbit serum. These tests appear to be reliable and a valuable tool in comparing the antibody response in various species of animals. The role of circulating antibodies in the various species is discussed briefly. The particular phase of this work has been brought to a logical conclusion and will be written up for publication. The tests themselves will be used as an assay tool in future experiments.

2. Clearance tests in immune rats indicate that the liver plays a smaller role in disposing of organisms in the blood than in mice. The relation between circulating antibodies and phagocytes is discussed. This work also has reached a conclusion and is to be written up for publication.

3. Experiments on Ascoli tests as a diagnostic aid are reported briefly. Further work is planned using the Ascoli test.

4. Experiments on the extraction of toxic substances from Bact. tularense have been negative and are being discontinued.

5. Low leucocyte and high leucocyte strains of inbred mice were found to be equally susceptible to infection with Bact. tularense.

6. Variation studies have shown that immunogenic and antigenic differences exist between smooth and non-smooth strains of Bact. tularense. Smoothness and virulence are associated in most cases but smooth avirulent strains also exist. Serum from both susceptible and non-susceptible species show smooth selective properties. Anti-serum prepared from resistant species retains its smooth selectivity to a greater extent than antiserum from susceptible species.

7. The progress of experiments on the isolation of polysaccharides and protein antigens is reported briefly.

ABSTRACT OF REPORT

January 1, 1953, to June 30, 1953

1. Mice are immunized to a slight degree only when injected with the purified polysaccharide from Bact. tularense.

2. The polysaccharide has an enhancing effect on the virulence of partially virulent strains.

3. Studies on the Ascoli antigen have shown it to be comparable to the polysaccharide in sensitizing red cells to hemagglutination, in use as a precipitin antigen and as a skin test antigen.

4. The polysaccharide, the Ascoli antigen, and red cells sensitized with polysaccharide induce an appreciable and comparable degree of immunity in white rats.

5. The comparative study of various killed culture vaccines indicates that smooth strains are more immunogenic than non-smooth strains and that ether and acetone killed cells are more effective than phenol killed, though none are as effective as the living cells which are partially virulent. The relation of immunogenicity to the Ascoli antigen is discussed.

6. Preliminary electrophoresis studies have indicated the presence of a component in the smooth virulent strain and smooth avirulent strain which is lacking in the non-smooth avirulent strains.

ANNUAL REPORT CONTRACT N6-ONR-26007

The following report constitutes an account of the work done during the period from January 1, 1953, through June 30, 1953, together with a brief summary of this work.

Routine matters such as virulence titrations, the preparations of vaccine, transfer of stock cultures, etc., are not reported. Detailed reports on work now in progress are not included.

PERSONNEL

Contract N6-ONR-26007 was activated July 1, 1951. The following personnel is listed, together with their status and time of employment.

Graduate Student Research Assistants	Employed
Lloyd Hendrix, Ph.D. candidate, Fall, 1954	July 1, 1951-June 30, 1953 1/2 time
Clifford Carlson, Ph.D. candidate, Spring, 1955	Feb. 9, 1953-June 30, 1953 1/2 time
Robert Atchison, M.A. candidate, Spring, 1954	June 1, 1953-July 31, 1953 1/2 time
Keith Long, Ph.D. candidate, Spring, 1955	June 1, 1953-July 31, 1953 full time
Technicians	
Mrs. Irene Dyke, L'ecole Rachel, Paris Lycee Brizeux, Quimper	Oct. 1, 1951-May 31, 1953 full time
Mrs. Dorothy B. Clark, Secretary	Apr. 15, 1953-June 30, 1953 3/4 time
Animal Care	
Harry Jeffrey	July 1, 1951-June 30, 1953 full time

* Note: Mrs. Irene Dyke is on leave of absence until September, 1953. Mrs. Mary Jane Taylor resigned December 31, 1952, and we were, therefore, short one

technician for the ensuing six months. Dr. Max Moody finished h Ph.D. degree and resigned December 31, 1953. He is now employed as a responsible investigator with the United States Public Health Service, Communicable Disease Center, Atlanta, Georgia. His work is concerned with Bacterium tularense and he is, therefore, continuing to profit by his training under ONR contract.

It is anticipated that Mrs. Carol Rausch will be added to the staff as full time technician beginning September 1.

MATERIALS AND METHODS

The following is a description of general materials and methods used. Whenever necessary, details are given with the separate experiments.

1. Glucose cysteine blood agar as described by Downs[1] is used for surface plate counts and for slants for carrying stock cultures. (Hereafter designated as GCBA).

2. Snyders broth is usually used as described by Snyder[2] for all liquid mixtures.

3. A standard suspension is defined as a suspension of such turbidity as to give a light transmission of 24 in the Coleman spectrophotometer 600 wave lengths. Such a suspension gives a plate count varying from 2 to 4 x 10^9 per ml, and is used as a starting suspension for all LD_{50} titrations so that an LD_{50} of 10^{-9} indicates that 1 to 2 organisms kill 50 per cent of the mice injected with 0.5 ml of the standard suspension. The Reed and Muench[3] method is used to calculate the LD_{50}. A standard suspension is hereafter designated as (SS).

4. The usual stock strains used are Sm virulent strain LD_{50} for mice $10^{-9.8}$ (SS).

Strain 38 completely avirulent for mice.

Strain Jap partially virulent for mice LD_{50} $10^{-3.8}$.

Strain Ince partially virulent for mice LD_{50} $10^{-7.0}$.

In cases where variants are used, the history of the variant is given.

5. Albino mice of approximately 20 gram weight predominantly male are used. These mice are purchased from the Maple Grove Rabbitry, Springfield, Missouri, and are healthy vigorous stock.

6. All agglutination tests are performed using Strain 38, killed with phenol, washed and suspended in saline to standard turbidity. Equal volumes of antigen and 2 fold dilutions of serum are mixed and incubated over night in the refrigerator for final reading the next day. The titer represents the highest dilution of serum at which definite agglutination may be seen with the naked eye and a concave mirror.

7. Hemagglutination tests were set up following the technique of Wright[4].

Cells: 5 to 7 ml type O human blood was added to 1 ml 2 per cent potassium oxalate, the suspension washed 3 times in 0.85 per cent saline, and the cells packed and resuspended to 10 per cent in 0.85 per cent saline.

Carbohydrates: The lyophilized carbohydrate was made up in a solution of 1 mg/ml in 0.85 per cent saline and kept frozen.

Sensitization: The carbohydrate solution was diluted to .005 per cent with 0.85 per cent saline and added to an equal volume of 10 per cent erythrocytes. This mixture was incubated 4 hours in a 37° water bath. At the end of the incubation period, the cells were washed four times in six or more volumes of 0.85 per cent saline and resuspended to 0.5 per cent in saline.

Agglutination: To 0.5 ml amounts of 2 fold serial dilutions of the serum, 0.5 ml of the 0.5 per cent cell suspension are added. The tubes are shaken and allowed to stand at room temperature for two hours. The test is read as the highest dilution of antiserum which shows 4+ agglutination (cells distributed uniformly over the bottom of the tube).

8. Ascoli tests

The antigens were prepared from tissues and organisms following methods given by Larson[8]. They are essentially aceton insoluble extracts from ether extracted heated tissues or organisms. These antigens give a precipitate when mixed with specific immune serum.

TECHNICAL REPORT

The work during the last 6 months has been somewhat handicapped by the loss of one full time technician and a Ph.D. candidate. One graduate student was added to the project on a half time basis, but his time has been spent in acquiring the basic techniques necessary in working with Bacterium tularense.

The experiments presented below deal largely with a study of antigens, the so-called Ascoli or heat stable antigen from tissues and from bacterial cells and the polysaccharide antigen which has been isolated from bacterial cells. We have shown repeatedly that mice can be solidly immunized only by the injection of living avirulent cells, and, although numerous methods have been attempted, no definitive evidence has been forthcoming of the presence of an immunizing antigen from the tissues which is not present in cells grown in cultures.

Antigenic differences have been demonstrated in this laboratory and elsewhere[6] between smooth and non-smooth strains of Bacterium tularense. These differences are apparent when living cells are used for immunizing mice, rabbits, and other animals.[7] In vitro serological tests yield very little evidence of these differences except in the higher titers of agglutinins produced in rabbits by the smooth strains. Larson's[5] work on the Ascoli antigen present in infected animals and in bacterial cells offers a suggestion that the Ascoli antigen may be used in certain diagnostic tests. Since it is present in tissues as well as in bacterial cells and since it is precipitable by acetone, a relationship to the polysaccharide obtainable from bacterial cells is suggested. The work presented below is part of a continuing study on the antigens of Bacterium tularense and their relation to the immunogenicity of the organisms and the relation of these antigens to the phenomenon of smooth-non-smooth variation.

A. The immunogenicity of polysaccharide and Ascoli antigens from Bacterium tularense.

1. Immunization of mice with polysaccharide.

Polysaccharide was prepared from Jap H cells as outlined on page 1 of the Appendix. Two groups of mice were injected with 2 doses of polysaccharide, 0.05 mg injected subcutaneously a week apart. One of the above groups received the same amount of polysaccharide suspended in Pendil (peanut oil) as an adjuvant. One group received Pendil alone. After two weeks the three groups were challenged as given in Table 1. It may be seen that there was some prolongation of life when Sm, the fully virulent strain, was used as a challenge. The strain Ince, having an LD_{50} of approximately 10^{-6}, is extremely unstable and, although it can be used as a challenge strain, the variability it exhibits makes it less suitable than a more stable strain. In this experiment the fact that mice receiving Pendil alone survived as well as those receiving the polysaccharide seems to preclude attaching any significance to the apparent protection given by the polysaccharide when Ince is used as challenge. The prolongation of life after Sm challenge is more significant because of the great virulence of this organism. If the somewhat arbitrary figure for the PR significance is set at 2, it may be seen that very little protection in mice resulted from the injection of the polysaccharide.

The polysaccharide used for mouse immunization was tested for serological activity by means of the hemagglutination tests. Jap H polysaccharide and a sample of known activity, Sm polysaccharide, were used to sensitize rabbit cells. The cells were sensitized by using .005 per cent solution of the respective polysaccharides and after sensitization were mixed with 2 fold serial dilutions of anti-Jap H serum from a rabbit which had recovered from an infection with this strain. The titer of the Jap H polysaccharide gave a hemagglutinating

Table 1

Protection of mice by the injection of Jap H polysaccharide

Immunizing agent	Challenge 100 LD_{50}	Results Dead/tested	ADD*	PR/
0.1 mgm, Jap H polysaccharide in saline	225 Sm**	11/11	7.73	1.8
	210,000 Ince	2/10	6.0	5.06
0.1 mgm, Jap H polysaccharide suspended in Pendil	As above Sm	10/10	7.9	1.8
	As above Ince	0/10	--	--
0.1 ml Pendil alone	As above Sm	10/10	4.6	1.07
	As above Ince	1/10	7.0	11.8
Normal mice	Sm	10/10	4.3	--
	Ince	9/10	5.3	--

* Average day of death.

/ PR = $\frac{\text{Mouse protection index of normal}}{\text{Mouse protection index of immune}}$

** 225 cells of the virulent strain Sm is equal to 100 LD_{50}.
210,000 cells of the less virulent strain Ince is equal to 100 LD_{50}.

titer of 1:20,480; the Sm polysaccharide, a titer of 1:-163,840. It is not unusual to obtain extremely high titers with cells sensitized with polysaccharides and in a personal communication Dr. Wright[6] tells me that this is true of the test in their hands also.

2. Immunization of mice with water insoluble material.

This water insoluble residue was essentially cellular debris remaining after sonic lysis. It contained 9.9 per cent N as determined by microkjeldahl and microscopically was amorphous. Mice given one intraperitoneal dose containing 0.8 mg (.079mgN) one week before challenge with 100 LD_{50} doses of virulent Bact. tularense (Sm) gave 60 per cent survival as against no survivals in the controls, and an average day of death of 8.5 against 4.5 in the controls, thus giving a PR of 3.16. It is apparent that mice were more adequately protected by this cellular debris than by the purified polysaccharide.

3. The effect of polysaccharide on virulence of a strain of low virulence.

The effect on virulence of pneumococcus polysaccharide is well known. Thus it seemed worth while to try to find out if the polysaccharide from a smooth virulent strain of Bact. tularense (Sm) would enhance the virulence of one of its rough avirulent clones (Sm_2). The LD_{50} of normal mice injected with 10 fold serial dilutions of Sm_2 was determined by injecting 6 mice per dilution at the levels shown in Table 2. At the same time, an LD_{50} was determined on the same series of dilutions of Sm_2 by injecting the organisms mixed with 0.5 ml of Sm polysaccharide containing 1.0 mg. This amount had been used repeatedly as an immunizing dose with no ill effects. As may be seen from the table the organism with the polysaccharide gave an LD_{50} of nearly 2 logs less than that given by the organism alone. The specificity of this effect will have to be determined.

Table 2

The effect of Sm polysaccharide on the LD_{50} of an avirulent clone (Sm_2)

Inoculum		Results					
Sm_2	Dilution used/	D/T	ADD	MPI	PR*	LD_{50}	Anti log of difference
Normal mice	10^{-5}	0/6	--	--	--		
0.5 ml organisms	10^{-4}	1/6	3.0	5.5	--		4.637
0.5 ml saline	10^{-3}	2/6	6.5	5.1	--	$10^{-2.67}$	-2.875
1.0 ml IP	10^{-2}	6/6	3.1	31.5	--		-antilog-
	10^{-1}	6/6	2.6	37.5	--		-57.81
Test mice, 0.5 ml organisms, 0.5 ml polysaccharide containing 1 mg.	10^{-5}	2/6	9.0	3.7	--		
	10^{-4}	5/6	5.4	15.4	0.36**		
	10^{-3}	6/6	2.8	35.3	0.14	$10^{-4.64}$	
	10^{-2}	6/6	2.17	46.1	0.68		
	10^{-1}	6/6	1.17	85.5	0.43		

/ Dilutions of standard containing 2 billion organisms/ml.

* PR = $\frac{\text{MPI normal}}{\text{MPI test}}$

** PR = 1 no protection
PR = <1 decreased resistance or enhanced virulence.

4. The sensitization of red cells by Ascoli antigen from mice.

In view of the fact that a delicate test for an antigen present early in an infection is very desirable we wished to find out how early the presence of the Ascoli antigen could be determined by means of the hemagglutination test. An earlier experiment had shown that mice infected with 2 to 20 organisms did not show a positive Ascoli test until the 4th day after infection when the Ascoli antigen was prepared from their tissues. In the present experiments, groups of mice were injected with 2, 20, 200, and 2000 virulent Bact. tularense. Two mice were killed each day and rabbit cells were sensitized by the Ascoli antigen from their tissues as explained in MATERIALS AND METHODS. As controls, rabbit cells were sensitized with Ascoli antigen prepared from bacterial cells and with polysaccharide. Table 3 shows that a relatively low titer of hemagglutination was obtained from mice killed on the fourth day and not from mice killed earlier. The high titers obtained by sensitization of cells with Ascoli antigen from bacteria and by sensitization of cells with polysaccharide would indicate that very little Ascoli antigen was present as early as the 4th day. This confirms earlier experiments and indicates that culture methods which are positive in 48 hours after inoculation of mice with infected material are a more rapid means of identification than the Ascoli test.

The above tests were repeated using Larson's[5] method for concentrating the Ascoli antigen from tissues, but no positive results were obtained earlier than the 4th day. That the Ascoli antigen could be concentrated by acetone precipitation was shown by a 4 fold increase in titer of the Ascoli antigen prepared from mice dead on the 4th day.

A comparison of 3 species of red cells sensitized by the Ascoli antigen

Wright[4] has shown that a large number of red cell species are suitable for use as a sensitized hemagglutinating antigen using polysaccharide from Bact.

Table 3

Hemagglutination tests using sensitized rabbit cells

Sensitizing antigen*	Source of antigen	Titer**
Mouse tissue Ascoli	Infected mouse killed after 24 hours	Negative
	48 hours	Negative
	72 hours	Negative
	96 hours	420
	5-7 days surviving mice injected with 2 to 20 organisms	Negative
Cellular Ascoli antigen		40,960
Polysaccharide coated cells		40,960

* Groups of 4 mice were injected with 2, 20, 200, 2000 virulent Bact. tularense. Two mice were killed each day and Ascoli antigen prepared from the tissues. Rabbit cells were sensitized by adding the mouse tissue antigen or the cellular ascoli or polysaccharide to the cells, and treating as set forth in materials and methods.

** The serum used was anti-tularense rabbit serum, agglutinating titer 1:10,240. The results of the Ascoli tests from the different lots of mice inoculated with varying numbers of organisms are pooled because of the negative nature of the results. All mice killed on the 4th day were positive, and the titer represents the average of the different levels.

tularense. In the following experiment rabbit, chicken, sheep, and human red cells were sensitized with an Ascoli antigen prepared from infected mice and with a polysaccharide prepared from Sm strain of Bact. tularense. A preliminary slide agglutination with unsensitized cells indicated the need for adsorption of natural agglutinins in rabbit serum. Chicken cells were agglutinated by both the normal and immune rabbit sera and were adsorbed before sensitization of the cells. The titers of the respective cells are given below and it may be seen that chicken and human cells are more suitable for use than sheep cells.

Serum	Cells	Titer	Polysaccharide
Jap H rabbit	Rabbit	12,560	40,960
	Chicken	12,560	156,000
	Sheep	1:20	156,000
	Human	12,560	156,000
Normal rabbit	Rabbit	--	--
	Chicken	--	--
	Sheep	--	--
	Human	--	--

5. The immunization of rats with Bact. tularense polysaccharide and Ascoli antigen.

Since Larson[8] showed that the Ascoli antigen induced the formation of antibody in rabbits, and since the polysaccharide showed some immunogenic capacity in mice as shown in this report, it seemed of interest to see if these antigens were immunogenic for rats. As shown in Table 4, 3 groups of rats of 40 each were inoculated with the respective antigens, 3 doses of 1 ml each

Table 4

Immunization of rats with polysaccharide and Ascoli antigen

Immunizing antigen	Dose	Challenge with Sm 0.5 ml Dead over tested				LD_{50}	Log Difference	PR*
		4×10^9	4×10^8	4×10^7	4×10^6			
A Ascoli antigen prepared from Sm cells	1 ml every other day for 3 doses	6/10	3/10	3/10	0/10	$10^{-1.59}$	+2.4	3.0
Controls No treatment		10/10	10/10	9/10	9/10	$>10^{-4}$		
B Sm polysaccharide containing 0.4 gamma/ml	As for A	9/10	6/10	6/10	1/10	$10^{-2.75}$	+1.4	3.0
Controls No treament		10/10	10/10	8/10	10/10	$>10^{-4}$		
C 5% rat red cells sensitized with Sm polysaccharide	As for A	8/10	7/10	2/10	2/10	$10^{-2.4}$	+1.6	3.0
Controls No treatment		10/10	10/10	8/10	7/10	$>10^{-4}$		

* The protective ratio was figured by averaging the total mortality in each group and the average day of death of all rats in each group. The percent mortality divided by the average day of death gives the mouse protective index. This figure for the vaccinated group divided into the MPI for the control groups gives a direct comparison which is termed the protective ratio.

intraperitoneally 3 days apart. The antigens were Ascoli antigen prepared from Sm cells, Sm polysaccharide, 0.4 gamma per ml, and five per cent rat cells sensitized with Sm polysaccharide. After 2 weeks the above 3 groups, together with 3 control groups, were challenged at 4 levels with 4 million, 40 million, 400 million, and 4 billion cells of virulent Sm. The 3 sets of controls were included because of the variability of the individual resistance of the rat. The rather drastic challenge was used for the same reason. The table shows that there was a distinct immunogenic effect shown by all of the antigens, the best protection being shown by the Ascoli antigen prepared from the bacterial cells. This is a crude antigen and probably contains other cellular antigens in addition to the polysaccharide. This experiment is being repeated with preparations in which the dosage is graded on the basis of total nitrogen so that a more quantitative comparison can be made.

The presence of Ascoli antigens in the rat tissues was tested for as the animals in the above experiment died. These results may be summed up briefly by saying that 52 per cent of the 111 control rats which died showed the presence of the Ascoli antigen in the liver and spleen, while 32 per cent of the immunized animals were positive. This is the usual finding in mice also, i.e., the immunized animals show a weak or negative Ascoli in contrast to the controls. This raises the intriguing question of whether the positive Ascoli is due only to the number of organisms in the infected animal or whether the immune animal secretes or destroys the antigen more rapidly than the normal animal.

6. <u>The use of polysaccharide and Ascoli antigens in the skin test</u>.

Persons who have had tularemia or occasionally a person who has been vaccinated repeatedly become exquisitely sensitive to a suspension of <u>Bacterium tularense</u>. Rabbits, and, to a lesser extent, guinea pigs become sensitive

after recovery from infection with a partially virulent strain. Recovered rats apparently do not become skin sensitive. In view of the interest which attaches to the various antigens which may play a role in infection in tularemia, it seemed worth while to test rabbits with the polysaccharide and Ascoli antigens. The regular skin test antigens, a formalinized suspension of a virulent strain, was used as a positive control, each antigen was controled negatively by the injection of saline containing the same diluent and preservative as the diluting fluid for the antigen. The material was injected into the shaven skin in 0.2 ml amount. The tests were read within an hour and each 12 hours thereafter. The results are summarized in Table 5. A positive test consisted of redness of 15 mm or more accompanied by swelling. No transient anaphylactic like reactions were seen.

Six guinea pigs which had recovered from an infection with Jap H gave only doubtful reactions with skin test antigen. Two normal control pigs were completely negative.

The above results indicate that the Ascoli and polysaccharide antigens yield positive skin tests in immune rabbits. Only one out of 3 rabbits vaccinated with killed cultures gave a weak positive with skin test antigen and Ascoli. The normal controls gave no reaction. There was no correlation between the degree of skin reactivity and the agglutination titer of the animal.

B. Comparative immunogenicity of vaccines made from variant strains of Bact. tularense.

We[9] have successfully used an avirulent strain as challenge in various neutralization and protective tests, since mice are so susceptible that the use of a highly virulent strain obscures any slight immunity which may be present. In this connection it is of interest to note that Bell, et.al,[10] have

Table 5

Skin tests in recovered, vaccinated, and normal rabbits

Number	Prior treatment and organism	Agglutinin titer	Skin test antigen and result			
			Skin test antigen/	Ascoli//	Sm polysaccaride///	Control*
1	Infection with avirulent Ri_1 smooth	5,120	+1-10	+1-10	—	—
2	Infection with Jap H	10,240	+1:1000	+1:1000	—	—
3	Infection with Ri_1	2,560	+1:100	+1:100	+1:10	—
5	Used for feeding lice infected with Sm	1,280	+1:1000	+1:100	+1:10	
6	Infection with 38	80	—	—	—	—
7	Infection with Jap H	5,120	+1:10	+ undil.	—	—
8	Infection with Ri_1	2,560	+1:100	+1:100	+ undil.	—
9	Vaccinated 3 x. 38 acetone vaccine	1,280	—	—	—	—
10	Vaccinated 3 x with Jap H acetone vaccine	640	—	—	—	—
11	Vaccinated 3 x with Ri_1 ether-extracted vaccine	1,280	+1:10	+ undil.	—	—
12	Normal	—	—	—	—	—
13	Normal	—	—	—	—	—
14	Normal	—	—	—	—	—

Antigens used:

/ Skin test antigen: formalinized suspension of cells killed with formalin. Final concentration 1:1000 HCOH..

// Ascoli antigen prepared by ether and heat extraction, then acetone precipitation of bacterial cells.

/// Sm polysaccharide 5 gamma per ml.

* Saline containing 1:1000 HCOH.

All antigens were injected undiluted, 1:10, 1:100, 1:1000. The highest dilution giving positive is designated in parenthesis.

shown that subcutaneous challenge of mice vaccinated with killed culture vaccine has served to indicate a considerable immunity in this difficultly immunizable animal, even when challenged with a fully virulent strain. When challenged with a strain of less virulence, the results were even more striking. Since there is at present no means of judging the potency of the tularemia vaccine used in man it seemed worth while to investigate the possibility that Larson's method of challenge would serve to indicate differences in potency between vaccines prepared in various ways and between vaccines made from smooth and non-smooth strains. Table 6, 7, and 8 sumarize a series of experiments designed to evaluate the various vaccines listed. Table 6 gives the comparison between vaccines made from Jap H, a smooth strain highly immunogenic in the living state, Sm_1, a smooth virulent clone, also immunogenic in the living state when the infection is controlled with streptomycin, and 38, predominantly non-smooth, avirulent, and slightly immunogenic. Suspensions containing 4 billion cells were made from each strain. In the case of Jap H, Sm_1, and 38 these were divided into 3 parts, one part was killed with ether, one with acetone, and one with 5 per cent phenol. Twenty-four mice were injected with 2 doses intraperitoneally, receiving a total of 8 billion cells. In addition, one group of 24 received 1 dose of living Jap H cells containing 40,000 cells. The group receiving living 38 received 4 billion cells in 2 doses. After 2 weeks each group of 24 mice, together with 2 comparable normal groups, were divided into 4 groups of 6 each and challenged subcutaneously with 1, 10, 100, and 1000 organisms of the virulent Sm_1 strain. This strain is of lessened virulence for rabbits and should have been comparable to Larson's strain 425 but was evidently too virulent for mice. It may be seen from Table 6 that the immunity of all vaccinated mice is slight as compared to the immunity of mice receiving living culture Jap H. As measured by Larson in terms of log difference

Table 6

Comparison of killed vaccines as immunizing agents in mice

Vaccine	Amount injected	Subcutaneous challenge Sm_1	LD_{50}	Log difference	PR
Jap H smooth, killed with ether	A dose of each vaccine containing 4 billion cells per ml.	All mice, including normal controls, received 0.3 ml of Sm_1 subcutaneously. The LD_{50} was calculated from deaths occurring when 6 mice were injected with 1, 10, 100, 1000 organisms respectively.	$10^{-7.2}$	2.3	3.5
Jap H, killed with acetone			$10^{-8.0}$	1.5	3.2
Jap H, killed with phenol			$10^{-8.1}$	1.4	1.8
Jap H, living culture	1 dose containing 40,000 cells		All survived		
Normal mice	No treatment		$10^{-9.6}$		
Sm_1 smooth, virulent	The vaccines were killed and administered as for the Jap H vaccines.	The challenge was the same as above.			
ether			$10^{-7.2}$	2.1	2.1
acetone			$10^{-8.6}$	2.7	5.3
phenol			$10^{-7.3}$	2.0	1.1
38 living	2 doses of 4 billion each		$10^{-8.8}$	0.5	1.3
Normal mice	No treatment		$10^{-9.3}$		
38 parent culture. Predominantly non-smooth, avirulent.	Vaccines were killed and administered as for the Jap H and Sm vaccines.	The challenge was the same as above.			
ether			$10^{-6.4}$	1.1	4.2
acetone			$10^{-7.0}$	0.5	2.6
phenol			$10^{-6.4}$	1.1	1.7
living	2 doses containing 4×10^9 cells each		$10^{-7.4}$	0.1	
Normal mice	No treatment		$10^{-7.8}$		

Table 7

A comparison of the immunizing capacity of ether-extracted cells, and supernate from various strains of Bact. tularense

Vaccine*	Amount injected	Intraperitoneal challenge Sm_1			Subcutaneous challenge		
		Challenge dose	LD_{50}	Log difference	Challenge dose	LD_{50}	Log difference
Sm_1 smooth, virulent cells, ether-extracted.	2 doses 4 x 10^9 cells	All mice, including the normal controls, received 0.5 ml of Sm_1 2 weeks after the last dose of vaccine. The LD_{50} was calculated from deaths occurring when 6 mice were injected with 2, 20, 200, 2000 organisms respectively.	$10^{-7.1}$	2.3	All mice received the vaccines as for those challenged intraperitoneally, but 2 weeks after last dose were challenged with 0.3 ml Sm_1 subcutaneously. Groups of 6 mice, including normal controls, were injected with 2, 20, 200, 2000 organisms respectively.	$10^{-7.3}$	1.1
Sm_1 supernate after ether-extraction	0.5 ml		$10^{-6.3}$	1.1		N D	--
Sm_2 rough, avirulent cells	As for Sm_1		$10^{-8.3}$	1.1		$10^{-8.5}$	-0.1
Sm_2 supernate	As for Sm_1		$10^{-8.6}$	0.6		$10^{-7.1}$	1.3
Ri_1 smooth, partially virulent cells	As for Sm_1		$10^{-7.5}$	1.9		$10^{-7.4}$	1.0
Ri_1 supernate	"		$10^{-7.8}$	1.6		$10^{-7.4}$	1.0
Ri_2 rough, avirulent cells	"		$10^{-8.5}$	0.9		$10^{-7.5}$	0.9
Ri_2 supernate	"		$10^{-7.0}$	2.4		$10^{-8.6}$	-0.2
38_3 smooth, avirulent cells	"		$10^{-6.8}$	2.4		$10^{-6.9}$	1.5
38_3 supernate	"		$10^{-7.6}$	1.6		$10^{-7.2}$	1.2
38_4 rough, avirulent cells	"		$10^{-6.3}$	0.9		N D	
38_4 supernate	"		10^{-9}	0.2		N D	
Normal mice	No treatment		$10^{-9.2}$			$10^{-8.4}$	

* The vaccines were prepared as given by Larson[6]. The cells were grown in liquid culture, separated, washed, and resuspended in saline and extracted with ether and centrifuged. The supernate was used after the removal of ether. Both cells and supernate were tested for sterility before use.

Table 8

Summary of a comparison of potency of vaccines

Vaccine and strain used*		Challenge Sm_1	Immunizing vaccines: Cells LD_{50}	Cells: Log difference	Supernatant LD_{50}	Supernatant: Log difference
Jap H ether	Partially virulent smooth	Subcutaneous in all cases	7.2	2.3		
Jap H acetone			8.0	1.5		
Jap H phenol			8.1	1.4		
Jap H living			0	—		
Sm_1 ether	Virulent smooth		7.2	2.1		
Sm_1 acetone			6.6	2.7		
Sm_1 phenol			7.3	2.0		
38 living	Predominantly non-smooth		8.5	0.5		
Controls			9.3	--		
38 ether	Predominantly non-smooth		6.4	1.1		
38 acetone			7.0	0.5		
38 phenol			6.2	1.3		
38 living			7.4	0.1		
Controls			7.5	—		
Sm_1 ether	Smooth	Intraperitoneal	7.1	2.3	8.3	1.1
Sm_2 ether	Non-smooth		8.3	1.1	8.8	0.6
Ri_1 ether	Smooth		7.5	1.9	7.8	1.6
Ri_2 ether	Non-smooth		8.5	0.9	7.0	2.4
Controls			9.4	--	—	—
38_3	Smooth	Subcutaneous in all cases	6.8	1.6	7.6	0.8
38_4	Non-smooth		8.3	0.1	9.0	-0.6
Sm_1	Smooth		7.3	1.1	ND/	--
Sm_2	Non-smooth		8.5	-0.1	8.1	0.3
Ri_1	Smooth		7.4	1.0	7.4	1.0
Ri_2	Non-smooth		7.5	0.9	8.6	-0.2
Controls			8.4	—	—	—

* The vaccines were prepared as shown in Table 7.
/ Not done.

between the LD_{50} of the test mice and the LD_{50} of the controls, our results are comparable to his when a fully mouse virulent strain is used for challenge. A summary of this experiment is given in Table 8. There is a suggestion here that an ether killed vaccine and possibly acetone vaccine is more potent than vaccines killed by phenol. Also that all vaccines made from the fully virulent strain (Sm_1) are more potent than one made from Jap H. The living 38 strain gave less protection than the killed vaccines and was not comparable to the living Jap strain.

Since Bell[10] uses an ether extraction for the preparation of vaccines an experiment was set up to determine how much protective substance was contained in the cellular portion and how much in the supernate. The organisms chosen for this experiment were smooth and non-smooth clones of virulent and avirulent organisms. The non-smooth clones were all of lessened virulence while the smooth clones, Sm and Ri, were as virulent as the parent culture, the smooth clone of 38 is entirely lacking in virulence. It might be expected that the use of vaccines made from such organisms would serve to indicate the association of an immunogenic antigen with the property of smoothness or virulence. The mice in groups of 24 were injected with the ether-extracted cells or with the aqueous supernate after ether extraction and removal of the cells. The methods for preparation of the vaccines is given in Table 7. In the case of Sm_1 and Sm_2, Ri_1 and Ri_2, and 38_3 vaccines, comparable groups of mice were challenged subcutaneously and intraperitoneally. Table 7 shows that with intraperitoneal challange the vaccines made from the cells of smooth clone Sm_1 and Ri_1 showed more protection as indicated by log difference than did the vaccines made from non-smooth Sm_2 and Ri_2. The same difference is apparent where subcutaneous challenge was used but the protection was not as great. In the case of vaccines made from the supernate, the smooth Sm_1 gave better protection than Sm_2, but the supernate

from Ri_2 gave better protection than the smooth Ri_1. When subcutaneous challenge was used, Ri_1 supernate gave better protection than Ri_2. This is an inconsistency which will have to be checked by further experiment.

Electrophoretic Analysis of Smooth and Rough, Virulent and Avirulent Strains of *Bacterium tularense*

The object of this experiment was to offer a direct comparison of the electrophoretic patterns of solutions containing water-soluble, non-dialysable components of representative smooth and rough clones of virulent and avirulent strains.

The following clones were selected:

Sm_1 smooth, virulent LD_{50} $10^{-9.5}$

Sm_2 rough, avirulent LD_{50} $10^{-2.8}$

38_3 smooth, avirulent $LD_{50} < 10^0$

38_4 rough, avirulent $LD_{50} < 10^0$

Colonies were picked from plates to slants to start the liquid cultures from which the cells used in the preparation of the samples were obtained. Small 100 ml liquid cultures were first started and after 10 hours incubation these were used to inoculate 500 ml cultures of each of the strains. After 18-20 hours incubation the cultures were centrifuged and the cells were re-suspended in saline and lysed in the Raytheon sonic oscillator for 15 minutes. The suspension was then centrifuged and the supernatants were dialysed against 4 liters of physiological saline 24 hours at 0-2°C.

Following dialysis, nitrogen determinations were made on the solutions. These results are given below. It was necessary to have a minimum of 1 per cent nitrogen.

After dialysis the first Sm_1 preparation, Sm_1I, was slightly contaminated with yeast. Therefore, another preparation was immediately begun (Sm_1II).

Similar preparations of Sm_2 were made. The first preparation contained too little nitrogen (Sm_2I) and, therefore, a second preparation was made (Sm_2II).

Preparations of 38_3 and 38_4 were prepared in a similar manner.

It was noted that there was much more cellular material remaining after sonic lysis of the rough strains (Sm_2, 38_4) than was obtained from the smooth strains (Sm_1, 38_3). These precipitates were treated for an additional 10 minutes by sonic oscillation, centrifuged, and the clear supernates combined with the original sonic supernates.

The nitrogen content of the samples listed below was determined by the micro-Kjeldahl method.

Sm_1I	0.80% N	38_3	1.37% N
Sm_1II	1.59% N	38_4	1.17% N
Sm_2I	0.35% N		
Sm_2II	0.88% N		

These samples were sent to the Department of Biochemistry for electrophoretic analysis. Technical difficulties with the apparatus were encountered, and the results were not entirely satisfactory and will have to be repeated. The following charts present the data obtained, however, and there was enough suggestion of difference in pattern between the rough and smooth forms to suggest that a component present in the smooth forms was lacking in the rough forms.

Although mobilities for the Sm_1I preparation cannot be determined because no visible pattern was obtained on the descending side and the starting boundary was omitted on the ascending side, it may be observed that the overall patterns for theSm_1I and Sm_1II samples are similar.

The general lack of similarity of the patterns of the two Sm_2 preparations can be attributed to (1) differences in initial protein concentration, (2) use of adifferent analytical cell, (3) differences in potential gradient due to changes in conductivity of the buffer, (4) slight differences in pH of the buffer.

The descending patterns of Sm_1II and Sm_2I offer some comparison. The smooth strain (Sm_1) has a considerable portion of material having mobilities from 4 to 6 x 10^{-8} which seems to be lacking in the rough Sm_2.

A similar comparison may be made between the descending patterns of 38_3 and 38_4 preparations. Material having mobilities of 2 to 5 seem to occur in significantly less degree in the rough 38_4 strain.

DISCUSSION

A study of antigens isolated from Bact. tularense presents difficult and interesting problems. These problems are analogous in some respects to those posed by P. pestis. In tularemia, as in plague, infection is necessary to produce a solid immunity; mice may be immunized by killed cultures of plague, but not guinea pigs. In tularemia rats may be immunized but not mice. Certain strains of plague (containing envelope) and tularemia (smooth, partially virulent) are more effective as live vaccines in an immunizable animal. We have shown that smooth, partially virulent strains are more immunogenic than non-smcoth strains in the living state, but the question of whether this immunogenicity rests in the ability of the organism to multiply in the ahimal has not been answered.

The studies reported here have to do with the question of the relation of the polysaccharide from Bact. tularense to immunity and the relation between this polysaccharide and the Ascoli antigen; thirdly, the relation between the Ascoli antigen and the effective immunizing antigen present in the infected animal; fourthly, the question of demonstrating a relation between smoothness and immunogenicity by the use of killed culture vaccines.

Our studies have shown that only a slight degree of immunity to 100 LD_{50} is produced in white mice by the injection of polysaccharide. In fact, the polysaccharide seems to enhance the virulence of a strain of tularense of lowered virulence. It seems possible that the polysaccharide may have been denatured by isolation or it may have been coupled with a protein in the cell, thus accounting for its lack of immunity in vivo. It also may be an antigen which is not reactive in the mouse in its isolated state. Experiments with rat immunization showed that Ascoli antigen from cells gave a significant degree of protection in this animal.

The relation of the Ascoli antigen from tissues or cells to the polysaccharide is not clear--both are acetone precipitable and it is possible that the Ascoli antigen contains the polysaccharide linked to a protein. Larson has shown that the Ascoli atigen induces antibody when injected into rabbits. We have confirmed this and have shown that it induces immunity in rats. Both polysaccharide and Ascoli may be used to sensitise red cells and as antigens in the precipitin tests and skin tests.

Coriell, Downs, et al[11] reported on the effectiveness of acetone precipitated vaccines and Ormsbee[12] has recently reported that solubilized antigens obtained by ether extraction of acetone dried cells are effective antigens in the white mouse.

In view of Bell's[10] report on the less drastic challenge by subcutaneous injection of a strain of slightly lowered virulence, it seemed worth while to initiate a comparison of killed vaccines prepared from smooth and non-smooth strains of Bact. tularense to see if antigenic differences could be measured in the white mouse. Various methods of killing were used with special emphasis on acetone precipitation and on ether extraction since these methods are effective in precipitating and extracting the Ascoli antigen. If we compare our results with those of Bell's, we see that in most cases the acetone and ether extracted vaccines give results comparable to his. It would seem that our strain used for challenge was somewhat more virulent. It is of great interest that smooth strains were more immunogenic than non-smooth strains and that even the smooth 38_3 strain was more immunogeic than the rough clone. In Table 6 and 7 where these comparisons were made, there are a few discrepancies, as for instance, in Table 7 where the 38_3 vaccines gave much better protection in the first experiment than in the second and where Ri_2 (rough) supernate gave greater protection than any of the smooth vaccines. These experiments are being repeated and

extended so as to determine the reliability of the method for the comparison and standardization of vaccines. Such a method of comparison would be very useful as at the present time the only measure of potency of the vaccine against tularemia which is a phenol killed culture is by means of injection into a rabbit with subsequent production of antibodies. The rabbit, in spite of antibody production, is not protected from infection and neither is the guinea pig or mouse. A virulent, predominantly smooth strain is used for the production of vaccine and such strains are good stimulators of antibody; but it does not necessarily follow that the vaccine is highly protective since a phenol killed culture of the avirulent 38 strain is also an antibody stimulator but is only slightly protective for the white mouse. We hope to show in future experiments that a real contrast may be shown between vaccines made from smooth and non-smooth cultures and between vaccines killed by various reagents.

The brief report on electrophoresis studies indicates the presence of an antigen in the smooth strains which is lacking in the non-smooth. The relation of this antigen to the Ascoli antigen is being investigated. Larson reported that after ether extraction the avirulent 38 strain yield less Ascoli antigen than the virulent strain. Since the parent strain of 38 is predominantly non-smooth and if the Ascoli antigen and the smooth antigen are related or identical, this might explain his results.

The work reported here may be summarized briefly as follows:

1. Mice are immunized to a slight degree only when injected with the purified polysaccharide from Bact. tularense.

2. The polysaccharide has an enhancing effect on the virulence of partially virulent strains.

3. Studies on the Ascoli antigen have shown it to be comparable to the polysaccharide in sensitising red cells to hemagglutination, in use as a precipitin antigen and as a skin test antigen.

4. The polysaccharide, the Ascoli antigen, and red cells sensitised with polysaccharide induce an appreciable and comparable degree of immunity in white rats.

5. The comparative study of various killed culture vaccines indicates that smooth strains are more immunogenic than non-smooth strains and that ether and acetone killed cells are more effective than phenol killed, though none are as effective as the living cells which are partially virulent. The relation of immunogenicity to the Ascoli antigen is discussed.

6. Preliminary electrophoresis studies have indicated the presence of a component in the smooth virulent strain and smooth avirulent strain which is lacking in the non-smooth avirulent strains.

APPENDIX

1. Polysaccharide fractionation of Jap H cells.
2. Electrophoretic pattern Sm_1 I and Sm_1 II.
3. Electrophoretic pattern Sm_2 I.
4. Electrophoretic pattern Sm_2 II.
5. Electrophoretic pattern 38_3 I.
6. Electrophoretic pattern 38_4 I.

Schematic for polysaccharide preparation from Bact. tularense (a modification of the method of Heidelberger, et al, (1950) for the preparation of the specific polysaccharide of the pneumococcus.

Sm cells grown in 3.1 liters medium dialyzed through cellophane during 18-24 hr. incubation. p.229

→ Culture chilled to 0-5°C. Equal vol. acetone added. Allowed to stand 24 hours. p.230

→ Centrifuged 4500 RPM. 45 min. Cells resuspended in D.W.

→ Lyophylized. Dry weight 7.8 grams.

Cells resuspended in D.W. and sonically lysed. Treated 1 hour (Bp 150, 9000 v/sec.) Raytheon Dsc-Hator Type R-22-3.

← Dialyzed against D.W. until el-free dialysate was obtained. p.232

← Centrifuged. 4500 RPM. 1 hr. p.236

↓ Water-insoluble residue discarded.

↓ Lyophylized water-soluble supernatant material.

5.5 gms. dry weight (p.237a)
9.25 % N (p.237d)
PR { 1.0 mg/mouse against 100 LD_{50} Sm = 2.16 (p.237c1)
0.1 " " " " " = 3.16 (p.237c2)

↓ 4.9 gms. redissolved in 5% acetate buffer PH 6.0 p.256

↓ Added equal vol. acetone. Allowed to stand in cold 24 hours.

→ Centrifuged. Redissolved in D.W. p.258

→ Deproteinized by adding 30 ml. $HCCl_3$ and 10 ml. Bu-OH per 100 ml. sol. and shaking two and ½ hours. p.258

← Centrifuged. Washed chloroform layer 2 D.W. Combined supernates.

← Added NaAc to 5% and HAc to PH 6.0 then equal vol. cold acetone. Allowed to stand in cold overnight. p.258

↓ Centrifuged. Ppt. redissolved in D.W. and dialyzed against D.W. Centrifuged 4500 RPM 1 hr. Supernatant lyophylized. p.261

1.7 gms. dry wt. (1st deprot.)
7.4 % N (p.237d)
PR { 1.0 mg/mouse against 100 LD_{50} Sm = 1.64
0.1 " " " " " = 2.08

↓ 1.0 gm. redissolved in D.W. and deproteinization procedure repeated using Warring blender ½ hour. p.281

↓ Ppt. (acetone; dialyzed) centrifuged; lyophylized.

0.7 gm. dry wt. (2nd deprot.)
6.65 % N (p.237d)

April 1, 1952

Polysaccharide Fractionation from Jap H Cells

Jap H cells from 10. liters 24 hour culture in medium dialyzed through cellophane during incubation.

→ Chilled to 0-6°C and equal vol. cold acetone added. Allowed to stand 24 hours.

→ Centrifuged in Sharples. Cells resuspended in D.W. P.272a

→ Suspension lyophylized. Dry weight cells = 9.0 gms.

8.0 gms. resuspended in 100. ml. D.W.

→ Cells sonically lysed ½ hour (Ep 150, 9000. ~/sec.) Raytheon oscillator P.272a.

→ Dialyzed against D.W. until Cl^--free.

→ Diluted to 400. ml. with D.W. Centrifuged 4500. P.P.M. 1. hour. Lysed ppt. ½ hour, longer, centrifuged, combined supernates. P.272a

→ Water-insoluble residue resuspended in D.W.

- 9.9 % N (p.272d)
- PR = 3.16 (p.272c) 0.8 $\frac{mg.}{mouse}$ one dose, 100 LD_{50} Sm

Water-soluble material in supernat ppt. by NaCl 1.% and equal vol. acetone.

→ Centrifuged. Redissolved ppt. in acetate buffer pH 6.0 Centrifuged.

→ Added equal vol. cold acetone to supernat. Allowed to stand 24 hours.

→ Centrifuged. Dissolved ppt. in D.W.

→ Deproteinized by adding 30. ml. $HCCl_3$ and 10. ml. Bu-OH per 100. ml. sol. and mixing for 5. min. intervals for total of 30. min. in Warring Blender.

→ Centrifuged. Washed chloroform layer with D.W. Combined supernates.

→ Added NaAc to 3.% and HAc to pH 6.0 and equal vol. cold acetone. Allowed to stand 24 hours.

→ Centrifuged. Dissolved ppt. in D.W. Dialyzed against D.W. 48 hours.

→ Centrifuged 4500. R.P.M. 1. hour. Supernatant lyophylized.

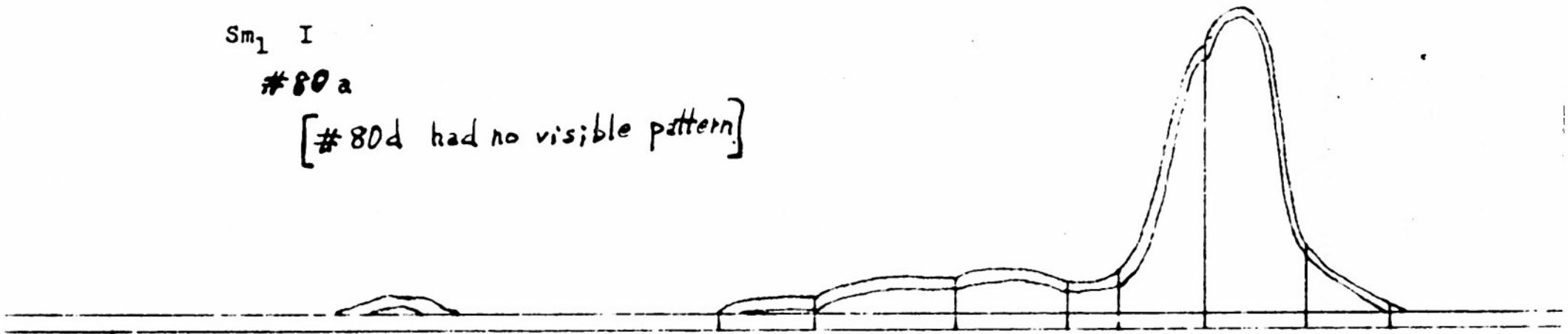
- 2.5 gms.
- 8.04 % N (p.272e)
- PR = 1.8 (p.272f) 0.1 mg. in 2 doses ≥ 100 LD_{50} Sm.

(Compare 2 P.283b)

Sm_1 I

#80 a

[#80d had no visible pattern]



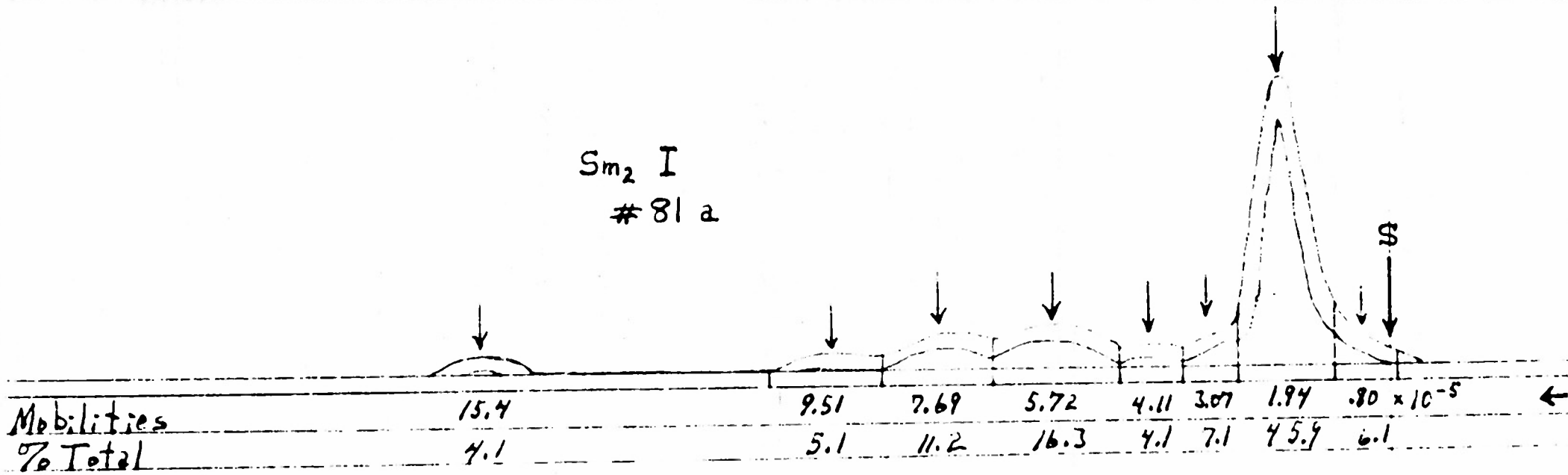
Mobilities — could not be calculated because starting boundry was not determined.

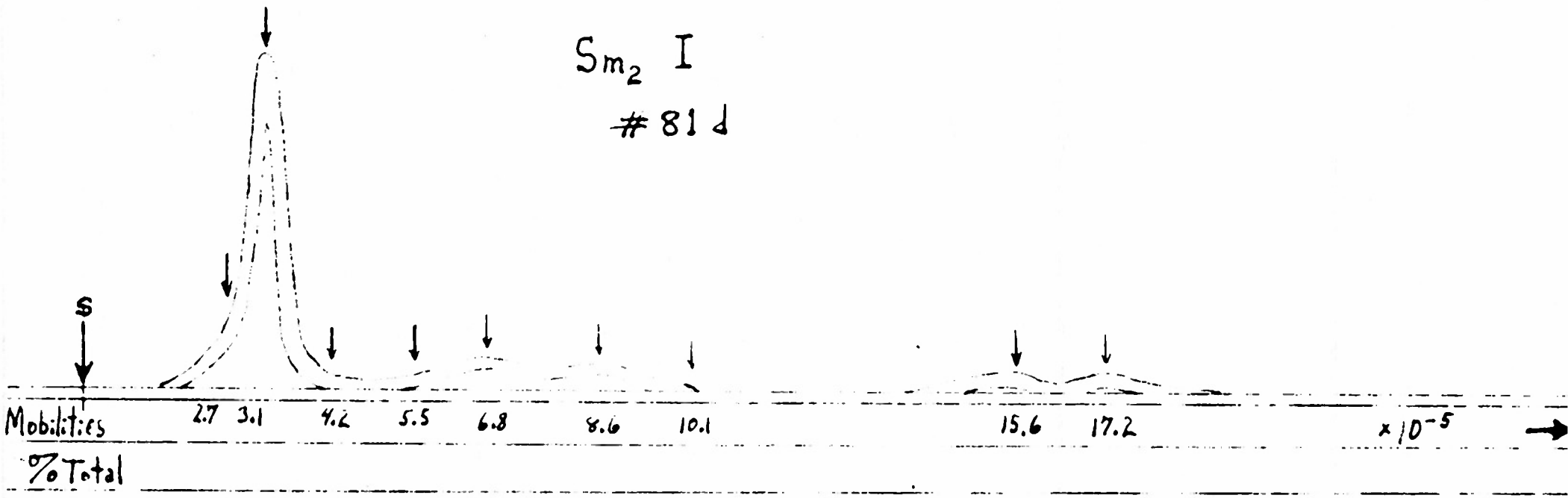
% Total	2.4		2.4	8.4	8.4	3.0	26.4	45.5	6.9

Sm_1 II [#84a had no visible pattern.]

#84d

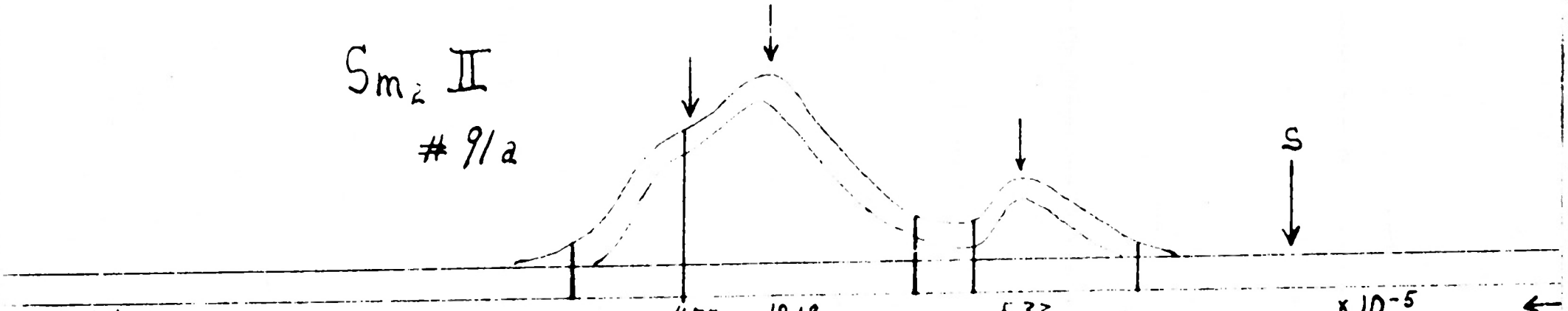
Mobilities	3.72	4.59	5.66	8.28	$\times 10^{-5}$ →
% Total	8.6	36.2	25.8	29.4	


Sm2 I
#81 a
S
Mobilities
% Total
15.4
9.51
7.69
5.72
4.11
3.07
1.94
.80 x 10-5
4.1
5.1
11.2
16.3
4.1
7.1
45.9
6.1

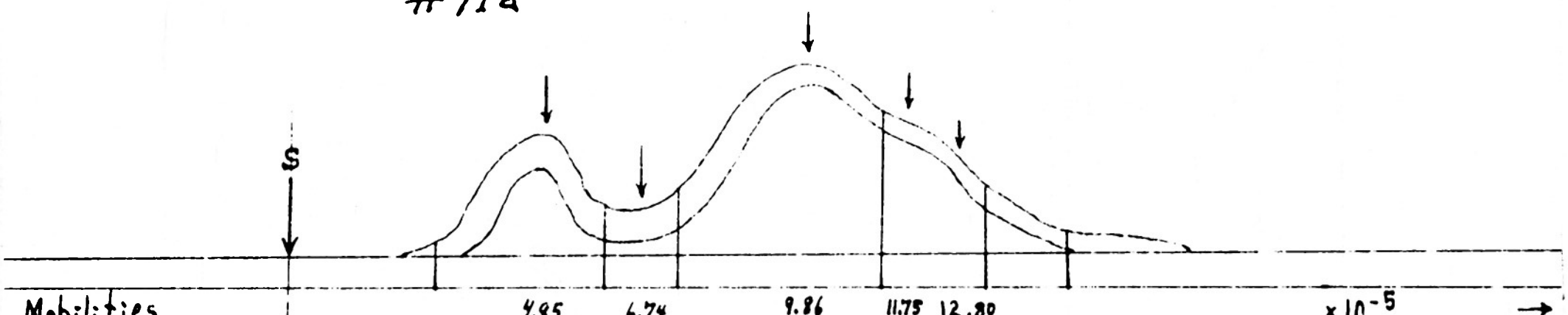

Sm2 I
#81 d
S
Mobilities
% Total
2.7
3.1
4.2
5.5
6.8
8.6
10.1
15.6
17.2
x 10-5

Sm₂ II

#91a

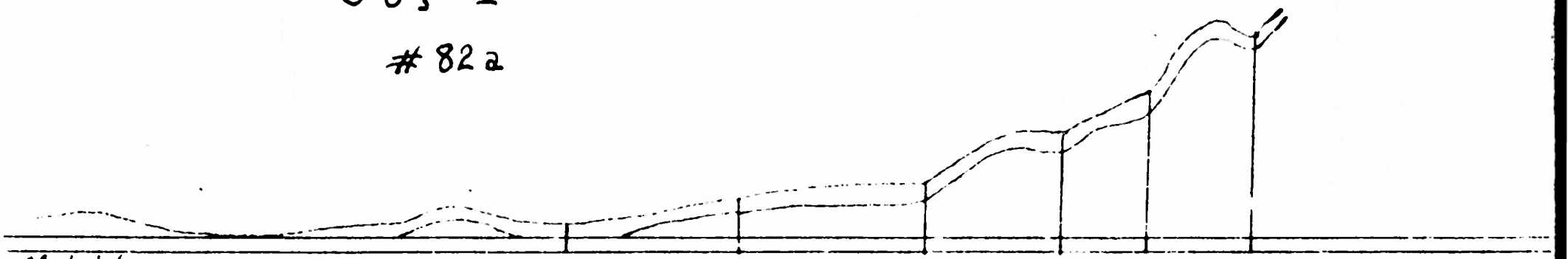


#91d



38_3 I

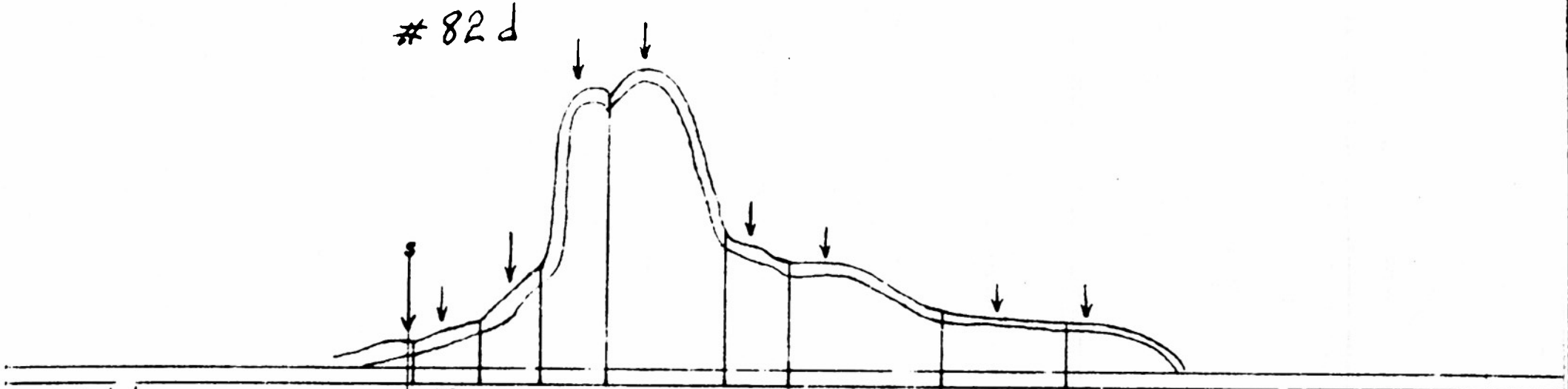
#82a



Mobilities — Could not be calculated because starting boundary was out of picture.

% Total

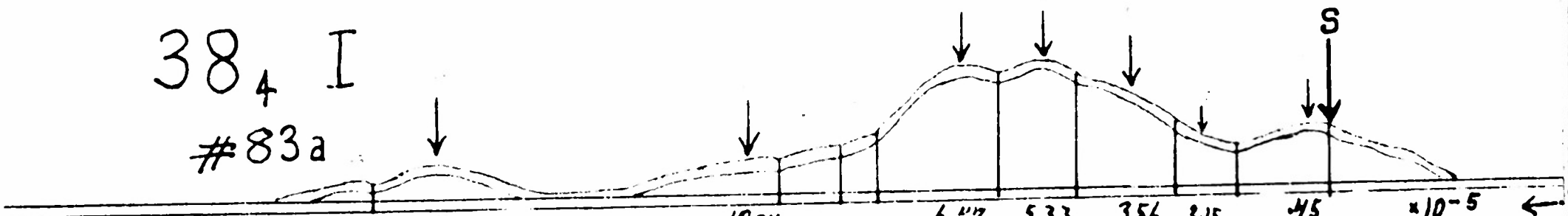
#82d



Mobilities	.49	1.6	2.58	3.68	5.4	6.55	9.33	10.75	$\times 10^{-5}$ →
% Total	2.9	5.3	18.4	34.2	9.2	16.2	8.1	5.5	

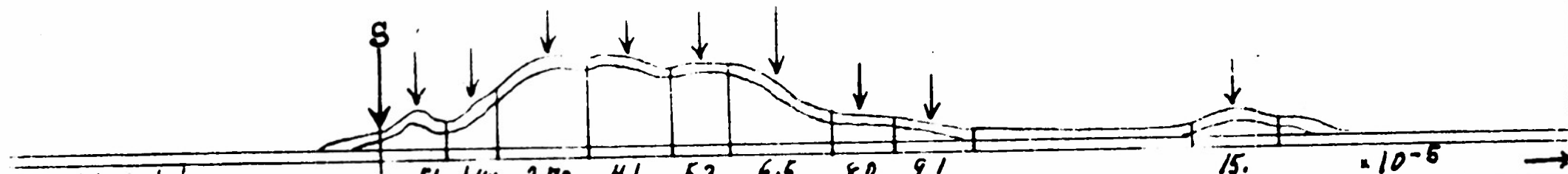
38_4 I

#83a



Mobilities		15.52		10.24	6.47	5.33	3.56	2.15	.45	$\times 10^{-5}$ ←
% Total	1.5	7.35	6.47	4.1	2.94	23.25	17.65	17.35	5.6	9.4	4.4

#83d



Mobilities	.56	1.44	2.70	4.1	5.3	6.5	8.0	9.1	15.	$\times 10^{-5}$ →
% Total	5.5	6.9	20.5	21.5	12.9	17.0	5.5	3.7	7.3	3.7

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